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Anthrax Toxin Components Stimulate Chemotaxis of Human Polymorphonuclear Neutrophils (42078)

BARBARA H. WADE, GEORGE G. WRIGHT, ERIK L. HEWLETT, STEPHEN H. LEPPLA,* AND GERALD L. MANDELL

*Division of Infectious Diseases, Department of Medicine, University of Virginia School of Medicine, Charlottesville, Virginia 22908, and * U.S. Army Medical Research Institute of Infectious Disease, Frederick, Maryland 21701*

Abstract. Effects of the three-component toxin of *Bacillus anthracis* on chemotaxis of human polymorphonuclear leukocytes (PMN) were investigated in an effort to determine the basis of the reported antiphagocytic effect of the toxin. The three toxin components, edema factor (EF), protective antigen (PA), and lethal factor (LF), were tested alone and in various combinations for their effect on PMN chemotaxis under agarose to formyl peptides and zymosan-activated serum. No component was active alone; combinations of EF + PA, LF + PA, and EF + LF + PA markedly stimulated chemotaxis (directed migration), but had little or no effect on unstimulated random migration. The toxin components were not themselves chemoattractants. EF in combination with PA had previously been identified as an adenylate cyclase in Chinese hamster ovary (CHO) cells. We found that EF + PA produced detectable cyclic adenosine 3′-5′monophosphate (cAMP) in PMN, but the level of cAMP was less than 1% of that produced in CHO cells by EF + PA, and in PMN by other bacterial adenylate cyclases. LF + PA (which stimulated chemotaxis to an equivalent extent) had no effect on cAMP levels. Thus, the enhancement of chemotaxis by anthrax toxin (at least by LF + PA) does not seem to be related to adenylate cyclase activity.

Anthrax toxin consists of three protein components: protective antigen (PA), edema factor (EF), and lethal factor (LF), each with a molecular weight of approximately 80,000 (1). PA is the major antigen involved in immunity to anthrax, and is the active component in nonviable anthrax vaccine (2). Intravenous administration of LF + PA produces death in many species of animals (3). EF + PA produces edema when injected into the skin. The complete toxin has antiphagocytic and antibactericidal effects, and enhances the virulence of attenuated strains of *Bacillus anthracis* (4). Recently, it has been found that EF is an adenylate cyclase, which is activated by a heat-stable eucaryotic protein, calmodulin (5). EF + PA produces high levels of cyclic adenosine 3′-5′monophosphate (cAMP) in Chinese hamster ovary (CHO) cells (5).

Chemotaxis, the directed migration of cells along a chemical gradient, has been studied extensively with polymorphonuclear neutrophils (PMN) and shown to be a receptor-mediated process that involves assembly and organization of microtubules and localization of microfilaments (6). Biochemical events that follow receptor binding include release of bound calcium, influx of external calcium, and activation of complex pathways involving production of arachidonate and its metabolism to produce hydroxyeicosatetraenoic acids, prostaglandins, and thromboxanes. Chemotaxis is depressed by cholera toxin (7), *Escherichia coli* enterotoxin (8), and urea extracts of *Bordetella pertussis* (9), all agents that promote formation of cAMP in phagocyte cells. Because of the adenylate cyclase activity of EF + PA, we hypothesized that the anthrax toxin also would inhibit chemotaxis of PMN, an effect that could contribute to the antiphagocytic activity observed by Keppie *et al.* (4). We observed instead a marked enhancement of chemotaxis.

Materials and Methods. *Toxin.* The three components were prepared as described previously (1), and resembled previous lots of biological and chemical properties. Stock solutions of the toxin components were stored at −70°C. *B. pertussis* urea extracts

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were prepared by the method of Confer and Eaton (9).

Chemotaxis. The agarose method of Nelson *et al.* (10) was used, with minor modifications described previously (8), except that PMN migration was measured from a magnified projection of the plates on the focusing screen of a microscope camera adaptor. Results are expressed as actual migration distance in millimeters, toward a well containing chemoattractant in the case of directed migration, toward an opposite well containing control buffer in the case of random migration. Agarose for electrophoresis, manufactured by Litex, Denmark, was used. Human peripheral blood neutrophils (PMN) were obtained by gravity sedimentation of heparinized venous blood. The PMN were incubated for 60 minutes at 37°C in a shaker waterbath with toxin components. The chemoattractant formylmethionyl phenylalanine (FMP) (Sigma Chemical Co.) was used at a concentration of 10^{-4} *M*. It was dissolved in dimethylsulfoxide and diluted 1:1000 in aqueous buffer; this avoided any effect of dimethylsulfoxide on chemotaxis. Limited experiments in which 10^{-7} *M* formyl-methionyl-leucyl-phenylalanine was used as chemoattractant produced similar effects (data not shown). Zymosan-activated serum was prepared as described by Ward *et al.* (11).

cAMP Production. PMN were obtained from normal humans by the gravity sedimentation procedure used in chemotaxis studies, or by Ficoll–Hypaque separation, dextran sedimentation, and hypotonic lysis of erythrocytes (12). PMN at a final concentration of 5×10^6/ml were incubated for 1 hr at 37°C with toxin components at 1 μg/ml, separately and in various combinations. Cells were sedimented by centrifugation and extracted with 0.1 *N* hydrochloric acid. The extracts were acetylated and cAMP determined by radioimmunoassay (13). Results are expressed as picomoles cAMP per 5×10^6 PMN. Similar results were obtained with the two types of cell preparation, and combined data are presented.

Results. Six experiments using PMN from four donors are summarized in Fig. 1. Open bars represent directed migration and hatched bars represent random migration. None of the components alone caused a significant change in either random or directed migration. In contrast, the combinations of EF + PA, LF + PA, and LF + EF + PA each caused significant increases in directed migration toward FMP. The presence of PA appeared to be an absolute requirement since cells treated with EF alone, LF alone, or the combination of EF and LF were not different from controls. The effects of EF + PA and LF + PA were not reversed by two washes of the toxin-treated PMN with Hanks' balanced salt solution prior to measurement of chemotaxis. There was no significant change in random migration with any of the components or combinations.

To determine whether the enhancement of chemotaxis was specific for chemoattractant peptides, experiments were performed using Zymosan-activated serum; results were similar to those obtained using FMP.

The effects on random and directed migrations of different concentrations of PA, EF + PA, LF + PA, and EF + LF + PA were explored to determine whether the stimulation of chemotaxis noted at 1 μg/ml was evident over a broad range of concentrations (Fig. 2). Stimulation of chemotaxis by the combinations evidently reached a maximum in the range 0.3 to 1 μg/ml. Further increase

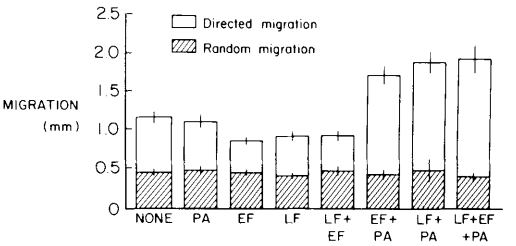

FIG. 1. Directed migration (open bars) and random migration (hatched bars) of human neutrophils after treatment with anthrax toxin components. Neutrophils, 5×10^6, suspended in 1.0 ml of Hanks' balanced salt solution containing 1 μg/ml each of one or more anthrax toxin components were incubated for 60 min in a 37°C waterbath shaker, then concentrated fivefold by centrifugation and allowed to migrate under agarose. Results are expressed as mm of migration from the edge of the well. The anthrax toxin components in each PMN preparation are shown beneath each bar. Directed migration was significantly enhanced by LF plus PA, EF plus PA, and LF plus EF plus PA ($P < 0.001$).

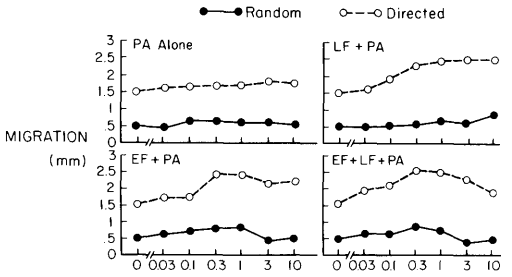

FIG. 2. Directed migration (upper curves) and random migration (lower curves) of human polymorphonuclear neutrophils incubated with a range of concentrations of anthrax toxin components indicated in the figure. The ordinates are least-square means of migration distances for three to six wells. Otherwise, experimental conditions were the same as for Fig. 1.

produced no change in the case of LF + PA, but appeared to decrease the response to EF + PA and EF + LF + PA. The changes in random migration, and the effects of PA alone were minor and were not significant in the more extensive experiments summarized in Fig. 1.

To determine whether the toxin components were themselves chemoattractants, PMN were put in the center well and their migration toward the outer well containing each of the anthrax toxin components alone or in combination was measured. Migration toward the toxin components did not exceed random migration.

Measurements of cAMP in PMN treated with toxin components, alone and in various combinations, are summarized in Table I. A concentration of 1 μg/ml was used for each component, since this had produced maximum effects on chemotaxis. *B. pertussis* urea extract was included as a positive control.

Only those combinations of components containing EF + PA produced appreciable elevation of cAMP. All cAMP elevations produced by anthrax toxin were <1% of those produced by urea extract from *B. pertussis*.

Discussion. The observed stimulation of chemotaxis (directed migration) but not of unstimulated random migration represents a new biological activity of the anthrax toxin which could provide clues to the nature of its antiphagocytic effect and its contribution to virulence of *B. anthracis*. The fact that the three toxin components were purified by repeated chromatographic procedures and approached homogeneity does not in itself eliminate the possibility that impurities were responsible for the observed effect. However, the absence of effects on chemotaxis by the individual toxin components makes this unlikely.

Early observations on the nature of this toxin tended to emphasize the requirement for cooperative action of the three components to produce lethal and antiphagocytic effects (4, 14), although PA + EF was evidently sufficient for production of cutaneous edema. More recent evidence has supported separate activities of EF and LF, which may be shown to compete for the sites of cell uptake formed by combination of PA with cell receptors (1). The present evidence that both EF + PA and LF + PA stimulate chemotaxis again raises the possibility that the three components act in a cooperative, or even a synergistic manner under appropriate conditions.

Exposure of PMN to EF + PA did not yield the high concentrations of cAMP observed after similar treatment of Chinese hamster ovary cells (5), nor the levels produced in PMN by *B. pertussis* urea extract as reported previously (9) and confirmed in the present work. The relatively low levels of cAMP produced in PMN were obtained at concentrations of EF + PA that produced maximum stimulation of chemotaxis. These

TABLE I. EFFECT OF ANTHRAX TOXIN AND *BORDETELLA PERTUSSIS* UREA EXTRACT ON cAMP LEVELS IN HUMAN PMN

Substance added to PMN[a]	pmole cAMP per 5×10^6 PMN
Control	3.41 ± 0.27
PA	2.90 ± 0.14
EF	4.37 ± 0.59
LF	2.50 ± 0.065
EF + PA	17.40 ± 1.26
LF + PA	5.48 ± 0.78
EF + LF + PA	10.10 ± 4.13
B. pertussis urea extract	>2000.00

[a] Concentration of anthrax toxin components was 1 μg/ml. Dilution of *B. pertussis* urea extract was 1:10.

low levels are consistent with the absence of inhibition of chemotaxis, but their relationship to the observed stimulation is not yet clear. The high levels of cAMP produced by pertussis extract abolished chemotaxis (9).

A possible basis for the observed stimulation of chemotaxis by the toxin is provided by observations that relate secretory products of PMN to modulation of chemotaxis (15, 16). The toxin may inhibit exocytosis or respiratory burst activity, interfere with the modulation process, and allow unrestrained chemotactic migration. Whatever the mechanism and pathophysiological significance of the observed stimulation of chemotaxis, anthrax toxin would appear to represent a useful probe for study of leukocyte chemotaxis.

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1. Leppla SH. *Bacillus anthracis* calmodulin-dependent adenylate cyclase: Chemical and enzymatic properties and interactions with eucaryotic cells. In: Greenyard P *et al,* eds. Advances in Cyclic Nucleotide and Protein Phosphorylation Research. New York, Raven Press, Vol 17:pp189–198, 1984.
2. Puziss M, Wright GG. Studies on immunity in anthrax X gel-adsorbed protective antigens for immunization of man. J Bacteriol **85**:230–236, 1963.
3. Lincoln RE, Walker JS, Klein F, Rosenwald AJ, Jones WI Jr. Value of field data for extrapolation in anthrax. Fed Proc **26**:1558–1562, 1967.
4. Keppie J, Harris-Smith PW, Smith H. The chemical basis of the virulence of *Bacillus anthracis IX:* Its aggressins and their mode of action. Brit J Exp Pathol **44**:446–453, 1963.
5. Leppla SH. Anthrax toxin edema factor: A bacterial adenylate cyclase that increases cyclic AMP concentrations in eukaryotic cells. Proc Nat Acad Sci USA **79**:3162–3166, 1982.
6. Schiffman E. Leukocyte chemotaxis. Ann Rev Physiol **44**:553–568, 1982.
7. Rivkin I, Rosenblatt J, Becker EL. The role of cyclic AMP in the chemotactic responsiveness and spontaneous motility of rabbit peritoneal neutrophils. J Immunol **115**:1126–1134, 1975.
8. Bergman MJ, Guerrant RL, Murad F, Richardson DW, Weaver D, Mandell GL. Interaction of polymorphonuclear neutrophils with *Escherichia coli.* J Clin Invest **61**:227–234, 1978.
9. Confer DL, Eaton JW. Phagocytic impotence caused by an invasive bacterial adenylate cyclase. Science (Washington, DC) **217**:948–950, 1982.
10. Nelson RD, Quie PG, Simmons RL. Chemotaxis under agarose: A new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. J Immunol **115**:1650–1656, 1975.
11. Ward PA, Cockrane CG, Muller-Eberhard HJ. The role of serum complement in chemotaxis of leukocytes in vitro. J Exp Med **122**:327–346, 1965.
12. Boyum A. Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest, **21**(Suppl 97):77–89, 1968.
13. Brooker G, Terasaki WL, Price MG. Gammaflow: A completely automated radioimmunoassay system. Science (Washington, DC) **194**:270–276, 1976.
14. Wright GG. Anthrax toxin. In: Schlesinger D, ed. Microbiology-1975. American Society for Microbiology, Washington, DC, pp292–295. 1975.
15. Gallin JI, Wright DG, Schiffmann E. Role of secretory events in modulating human neutrophil chemotaxis. J Clin Invest **62**:1364–1374, 1978.
16. Clark RA. Extracellular effects of the myeloperoxidase-hydrogen peroxide-halide system. In: Wassermann G, ed. Advances in Inflammation Research. New York, Raven Press, Vol 5:pp107–146, 1983.

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